PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C07K 15/28, C12P 21/06, A61K 39/395, C12N 15/00, 1/00, 5/00, C07H 15/00	A1	(11) International Publication Number: WO 94/14847 (43) International Publication Date: 7 July 1994 (07.07.94)

(21) International Application Number: PCT/US93/12339

(22) International Filing Date: 17 December 1993 (17.12.93)

(30) Priority Data:
07/996,675 24 December 1992 (24.12.92) US

(71) Applicant: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 300 Lakeside Drive 22nd floor, Oakland, CA 94612 (US).

(72) Inventors: ZANETTI, Maurizio; 6112 La Jolla Hermose Avenue, La Jolla, CA 92037 (US). BILLETTA, Rosario; 359 Coast Boulevard South, La Jolla, CA 92037 (US).

(74) Agents: DREGER, Walter, H. et al.; Flehr, Hohbach, Test, Albritton & Herbert, 4 Embarcadero Center, Suite 3400, San Francisco, CA 94111-4187 (US).

(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: GENETICALLY ENGINEERED IMMUNOGLOBULINS

(57) Abstract

This invention relates to the introduction of oligopeptide epitopes of biological receptor, preferably herein the CD4 receptor, for expressing within the three dimensional fold of an immunoglobulin (Ig) molecule, thus creating molecules useful to induce specific, biologically active anti-receptor immunity.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria
AU	Australia
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CS	Czechoslovakia
CZ	Czech Republic
DE	Germany
DK	Denmark
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgystan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakhstan
LI	Liechtenstein
LK	Sri Lanka
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SI	Slovenia
SK	Slovakia
SN	Senegal
TD	Chad
TG	Togo
TJ	Tajikistan
TT	Trinidad and Tobago
UA	Ukraine
US	United States of America
UZ	Uzbekistan
VN	Viet Nam

GENETICALLY ENGINEERED IMMUNOGLOBULINS

Cross Reference to Related Applications

This is a continuing application of applications U.S. Serial No. 07/947415 and U.S. Serial No. 07/947521, both filed 18 September 1992, each being a continuation application of application U.S. Serial No. 07/316144, filed 24 February 1989.

Field of the Invention

The present invention may utilize in its preferred embodiments, the use of recombinant DNA technology to genetically engineer natural or synthetically-derived immunoglobulin molecules, imparting therein novel epitopes, so as to create novel entities that can be employed *in vitro* and *in vivo* in a variety of means, such as to immunize against pathogens, and for example, build tolerance to antigens.

In preferred embodiments, the epitopes are inserted into the so-called heavy or light chain variable domain of a given immunoglobulin molecule. Thus, known recombinant DNA technologies come to bear in the present invention, helping create novel immunoglobulin entities that retain functionality by localizing to particular cell types mechanistically via the so-called constant domains but otherwise functionally exploited to provide a novel

localization of a particular antigenic determinant or epitope.

Background of the Invention

Recombinant DNA technology has reached the point currently of being capable, in principle, of providing the methodology sufficient to identify, isolate and characterize DNA sequences, configure them for insertion into operative expression vectors and transfect those vectors variously into recombinant hosts such that those hosts are harnessed in their ability to produce the polypeptide encoded by the DNA sequence. Obviously, many variations attend the methodology associated with recombinant DNA technology, and particular means are not without inventive faculty. Nonetheless, methods are generally known in the published literature enabling requisite mental equipment for the art skilled to practice recombinant DNA technology in the production of polypeptides from a given recombinant host system.

Immunoglobulins (Igs) are the main effectors of humoral immunity, a property linked with their ability to bind antigens of various types. In view of the myriad numbers of antigens to a particular host organism, it can be appreciated that there are a like number or more of immunoglobulins that contain antigenic determinants or epitopes against particular such antigens.

Immunoglobulin molecules are unique in their functionality of being capable of localizing to certain cell types, probably by means of mutual recognition of certain receptors that are located on the cell membrane. Immunoglobulins demonstrate a second general property whereby they act as endogenous modulators of the immune response. Igs and their idiotypic determinants have been used to immunize at the B- and/or T-cell level against a variety of exogenous antigens. In many cases, the

immunity they evoke is comparable with that induced by the antigen itself. Although the principle underlying this phenomenon is understood, little is known about the molecular basis and the minimal structural requirements for the immunogenicity of Igs molecules and the interaction between those regions which may be responsible for such immunogenicity and the regions that are thought to provide the localization of a given immunoglobulin molecule with a particular cell/receptor type.

In the last many years, much progress has been made in endeavors to understand the immunogenic properties, structure and genetics of immunoglobulins. See Jeske, *et al.*, Fundamental Immunology, Paul, ed., Raven Press, New York (1984), p 131 and Kabat, Journal Immunology 141, 525 (1988).

Initially, the antigenicity of the so-called variable (V) domain of antibodies was demonstrated. Oudin, *et al.*, Academy of Sciences D 257, 805 (1963) and Kunkel, *et al.*, Science 140, 1218 (1963). Subsequently, further research pointed out the existence of discrete areas of variability within V regions and introduced the notion of hypervariable (HV) or complementarity-determining regions (CDR). Wu, *et al.*, J. Exp. Med. 132, 211 (1970). Many studies since have indicated that the immunogenic property of Ig molecules is determined presumably primarily by amino acid sequence contained in the CDRs. Davie, *et al.*, Ann. Rev. Immunol. 4, 147 (1986).

The basic immunoglobulin or antibody structural unit is well understood. The molecule consists of heavy and light chains held together covalently through disulfide bonds. The heavy chains are also covalently linked in a base portion via disulfide bonds and this portion is often referred to as the so-called constant region which is thought responsible for a given immunoglobulin molecule being mutually recognizable with certain sequences found

at the surface of particular cells. There are five known major classes of constant regions which determine the class of the immunoglobulin molecule and are referred to as IgG, IgM, IgA, IgD and IgE. The N-terminal regions of the so-called heavy chains branch outwardly in a pictorial sense so as to give an overall Y-shaped structure. The light chains covalently bind to the Y branches of the two heavy chains. In the regions of the Y branches of the heavy chains lies a domain of approximately 100 amino acids in length which is variable, and therefore, specific for particular antigenic epitopes incidental to that particular immunoglobulin molecule.

It is to the Y branches containing the variable domains harboring the antigenic epitopes to which the particular attention is directed as a predicate of the present invention.

Prior researchers have studied and manipulated entire CDRs of immunoglobulins, producing chimeric molecules that have reported functionality. Exemplary attention is directed to Jones, *et al.*, Nature 321, 522 (1986) reporting on a V-region mouse-human chimeric immunoglobulin molecule. This research thus amounted to a substantially entire CDR replacement as apparently does the research reported by Verhoeyen, *et al.*, Science 239, 1534 (1988); Riechmann, *et al.*, Nature 332, 323 (1988); and by Morrison, Science 229, 1202 (1985). See also European Patent Application Publication No. 125023A, published 14 November 1984.

Bolstered by the successful research summarized above that resulted presumably in functional chimeric molecules, the goal of the present research was to explore further the variable region contained in the N-terminus Y branches. It was a goal of the present research to manipulate these variable regions by introduction or substitution of novel determinants or epitopes so as to create novel immunoglobulin molecules that would possibly retain the

localization functionality and yet contain functional heterologous epitopes. In this manner, the novel immunoglobulin molecules hereof could be employed for use within the organism at foreign sites, thereby imparting immunity characteristics in a novel site-directed manner.

A problem facing the present researchers at that time lay in the fact that epitopes are found in a region of the Y branch. Therefore, it was difficult to envision whether any manipulation of the variable region would be possible without disrupting the interaction of heavy chain with the corresponding light chain, and if that proved inconsequential, whether the resultant molecule would retain its functionality, with respect to the novel epitope, in combination with the constant region of the basic immunoglobulin molecule. Thus, even hurdling the problem of where to experiment, it was not possible to predict whether one could successfully produce such novel, bifunctional immunoglobulin molecules.

The present research and invention are based upon the successful threshold experiment, producing model, novel immunoglobulin molecules found to be fully functional by virtue of their ability to localize on certain cell/receptor sites and elicit reactivity to the antigens specific for the introduced novel antigenic determinant or epitope.

<u>Summary of the Invention</u>

The present invention is based upon the successful production of novel immunoglobulin molecules having introduced into the N-terminus variable region thereof a novel epitope not ordinarily found in the immunoglobulin molecule used as a starting molecule.

Expression of oligopeptide epitopes in the hypervariable loops of an antibody molecule, antigenization of antibody,

is an efficient procedure for stabilizing oligopeptides within a limited spectrum of tertiary structures. As a result, peptides acquire an ordered conformation, and antigenized antibodies (^{Ag}Ab) can serve as useful mimics of antigens and ligands.

More particularly, this invention relates to the introduction of oligopeptide epitopes of biological receptor, preferably herein the CD4 receptor, for expression within the three-dimensional fold of an immunoglobulin (Ig) molecule, thus creating molecules useful to induce specific, biologically active anti-receptor immunity.

The present invention is thus directed to novel immunoglobulin molecules having at least one novel heterologous epitope contained within the N-terminus variable domain thereof, said novel immunoglobulin molecule having retained functionality with respect to its C-terminus constant domain of the heavy chain specific for a particular cell/receptor type, and having novel, specific epitope *in vitro* and *in vivo* reactivity.

The present invention is further directed to pharmaceutical compositions containing, as essential pharmaceutical principle, a novel immunoglobulin hereof, particularly those in the form of an administrable pharmaceutical vaccine.

The present invention is further directed to methods useful for building tolerance to certain antigens, including those associated with autoimmune diseases, or for down-regulating hypersensitivity to allergens, or for providing active or passive immunity against certain pathogenic antigens, by administering to an individual in perceived need of such, a novel immunoglobulin molecule as defined above.

The present invention is further directed to novel recombinant means and methods useful for preparing, identifying and using the novel immunoglobulin molecules hereof including DNA isolates encoding them, vectors operatively harboring such DNA, hosts transfected with such vectors, cultures containing such growing hosts and the methods useful for preparing all of the above recombinant aspects.

The new approach to generate protective immunity, antigenized antibodies, aims at interfering with the mechanisms of virus binding and cell adhesion. ^{Ag}Abs is a new way to express immunologically-relevant oligopeptides based on the exploitation of hypervariable (HV) loops as a privileged site for the maintenance of conformation and three-dimensionality. HV loops are convex sites with the ability to provide contact bonds of the ionic, hydrogen, and van der Waals types for receptors, ligands and antibodies.

The use of ^{Ag}Abs is an excellent vehicle for immunization, possibly due to its intrinsic ability to conserve and/or confer tertiary structure to oligopeptides, an important feature to antigenicity and immunogenicity.

Among the advantages over conventional approaches, ^{Ag}Abs combines molecular specificity (amino acid sequence) with three-dimensionality (the Ig-fold)-characteristics of key importance to antigenicity and immunogenicity - and the ability to target the target antigen presenting cells directly (APCs) via the Fc portion of the molecule. In our hands, ^{Ag}Abs elicits immunity against the native antigen even across the boundaries of major histocompatibility complex (MHC) restriction.

It should be pointed out that although the emphasis of this application will be on active immunization, monoclonal antibodies of novel specificity and exquisite

biological function that may be generated in the course of these studies may be considered for passive therapy as well to complement active immunization.

Detailed Description of the Invention

The present invention is described herein with particular detail for the preparation of model, novel immunoglobulin entities. This description is provided, as it was conducted, using recombinant DNA technology. Further detail herein defines methods by which one can test a given immunoglobulin to assure that it exhibits requisite functionality common to its starting material immunoglobulin and specially as to its novel epitopic antigenic activity. Given this information with respect to the particular novel immunoglobulin molecules described herein, coupled with general procedures and techniques known in the art, the art skilled will well enough know how to configure recombinant expression vectors for the preparation of other novel immunoglobulin molecules falling within the general scope hereof for use as herein described. Thus, having described the threshold experiment of the successful preparation of a novel immunoglobulin molecule, one skilled in the art need not follow the exact details used for reproducing the invention. Instead, the art skilled may borrow from the extant, relevant art, known techniques for the preparation of still other novel immunoglobulin molecules falling within the general scope hereof.

1. Figure Legends

Figure 1 is a diagram illustrating the construction of the pNy1NANP expression vector.

Figure 2 is an SDS-PAGE of the y1NANP and WT recombinant Ig.

Figure 3 shows the binding of ^{125}I-labelled monoclonal antibody Sp-3-B4 to engineered antibody γ1NANP.

Figure 4 is a Western blot binding of ^{125}I-labelled antibody Sp3-B4 to engineered antibody γ1NANP and localization of the engineered $(NANP)_3$ epitope in the H chain.

Figure 5 shows results of cross-inhibition of ^{125}I-labelled antibody Sp3-B4 binding to synthetic peptide $(NANP)_3$ (panel A) or engineered antibody γ1NANP (panel B) by γ1NANP Ig or peptide $(NANP)_3$.

Figure 6 is a stereo drawing of the α carbon backbone of the first (V1) extracellular domain of human CD4 (residues 1-98) from the x-ray crystal analysis.

Figure 7 depicts structure and epitope expression of ^{Ag}Abs expressing oligopeptides of human CD4 and reactivity with an anti-CD4 antibody.

Figure 8 shows C57BL/6 mice immunized with $\gamma 1CD4^{B}$ (100μg/injection) intraperitoneally at monthly intervals first in complete Freund's adjuvant and subsequently in incomplete adjuvant. Hybridomas were prepared from a mouse with the highest titer of inhibitory antibodies by fusing with Sp2/0 myeloma cells. Syncytia assay conditions and percent inhibition were described in the text.

Figure 9 provides an analysis of mAbPL1. Upper panel: Dose-response inhibition of syncytia formation. Lower panel: FACS analysis and surface staining of CD4 on CEM cells.

Figure 10 is a Western Blot on recombinant (γ)CD4. 2 μg of soluble rCD4 were loaded onto a 10% SDS/PAGE gel, electrophoresed and transferred to PVDF membrane

(Millipore). After blotting, the membrane was blocked by soaking in 5% dry milk in PBS and incubated with 10μg/ml of each mAbPL overnight at 4 °C. Binding was revealed with a HRP-conjugated goat antibody to mouse Ig (γ-chain specific) in PBSA. The OKT4D and a noncorrelate Ab have been used as a positive and negative control, respectively.

2. General Methods and Definitions

"Expression vector" includes vectors which are capable of expressing DNA sequences contained therein, where such sequences are operatively linked to other sequences capable of effecting their expression. It is implied, although not always explicitly stated, that these expression vectors may be replicable in the host organisms either as episomes or as an integral part of the chromosomal DNA. "Operative," or grammatical equivalents, means that the respective DNA sequences are operational, that is, work for their intended purposes. In sum, "expression vector" is given a functional definition, and any DNA sequence which is capable of effecting expression of a specified DNA sequence disposed therein is included in this term as it is applied to the specified sequence. In general, expression vectors of utility in recombinant DNA techniques are often in the form of "plasmids" referred to as circular double stranded DNA loops which, in their vector form, are not bound to the chromosome. In the present specification, "plasmid" and "vector" are used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which become known in the art subsequently hereto.

Apart from the novelty of the present invention involving the introduction of novel epitopes by means of repositioning or augmentation of a parent immunoglobulin,

it will be understood that the novel immunoglobulins of the present invention may otherwise permissively differ from the parent in respect of a difference in one or more amino acids from the parent entity, insofar as such differences do not lead to a destruction in kind of the basic activity or bio-functionality of the novel entity.

"Recombinant host cells" refers to cells which have been transfected with vectors defined above.

Extrinsic support medium is used to support the host cells and includes those known or devised media that can support the cells in a growth phase or maintain them in a viable state such that they can perform their recombinantly harnessed function. See, for example, ATCC Media Handbook, Ed. Cote *et al.*, American Type Culture Collection, Rockville, MD (1984). A growth supporting medium for mammalian cells, for example, preferably contains a serum supplement such as fetal calf serum or other supplementing component commonly used to facilitate cell growth and division such as hydrolysates of animal meat or milk, tissue or organ extracts, macerated clots or their extracts, and so forth. Other suitable medium components include, for example, transferrin, insulin and various metals.

The vectors and methods disclosed herein are suitable for use in host cells over a wide range of prokaryotic and eukaryotic organisms.

"Heterologous" with reference herein to the novel epitope for a given immunoglobulin molecule refers to the presence of (at least one) such epitope in the N-terminus domain of an immunoglobulin that does not ordinarily bear that epitope(s) in its native state. Hence, that chain contains heterologous epitope sequence(s). Such heterologous epitope sequences shall include the classic antigenic epitopes as well as receptor binding domains or binding

regions that function as receptor sites, such as the human CD4 binding domain for HIV, hormonal receptor binding ligands, retinoid receptor binding ligands and ligands or receptors that mediate cell adhesion.

"Chimeric" refers to immunoglobulins hereof, bearing the heterologous epitope(s), that otherwise may be composed of parts taken from immunoglobulins of more than one species. Hence, a chimeric starting immunoglobulin hereof may have a hybrid heavy chain made up of parts taken from corresponding human and non-human immunoglobulins.

In addition to the above discussion and the various references to existing literature teachings, reference is made to standard textbooks of molecular biology that contain definitions and methods and means for carrying out basic techniques encompassed by the present invention. See, for example, Maniatis, *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1982 and the various references cited therein, and in particular, Colowick *et al.*, Methods in Enzymology Vol 152, Academic Press, Inc. (1987). All of the herein cited publications are by this reference hereby expressly incorporated herein.

The foregoing description and following experimental details set forth the methodology employed initially by the present researchers in identifying and characterizing and preparing particular immunoglobulins. The art skilled will recognize that by supplying the present information including the wherewithal of the location and makeup of the epitope containing domain of a given immunoglobulin, and how it can be manipulated to produce the novel immunoglobulins hereof. Therefore, it may not be necessary to repeat these details in all respects in their endeavors to reproduce this work. Instead, they may choose to employ alternative, reliable and known methods, for example, they may synthesize the underlying DNA

sequences encoding a particular novel immunoglobulin hereof for deployment within similar or other suitable, operative expression vectors and culture systems. Thus, in addition to supplying details actually employed, the present disclosure serves to enable reproduction of the specific immunoglobulins disclosed and others, and fragments thereof, such as the individual chains for *in vitro* assembly, using means within the skill of the art having benefit of the present disclosure. All of such means are included within the enablement and scope of the present invention.

3. Description of Particularly Preferred Embodiments

Research focused on the engineering, expression and testing of antibodies containing discrete sequences of human CD4. The four regions used for "antigenization" are listed below (Table I) and are also depicted in Figure 6 in the context of three-dimensional folding of CD4. The first two sequences used correspond to the putative HIV-binding site were 42-49 and 41-55. The two new sequences correspond to amino acid residues 38-49. Among the latter, one is the wild type (WT) sequence, the other contains a mutation glutamine (Q) to alanine (A) in position 40 that according to published data (24) possesses significantly increased affinity for gp120. In each instance, these oligopeptide sequences were inserted in the CDR3 loop of a murine heavy chain V region gene expressed along with a human $\gamma 1$ constant region gene to yield a chimeric (mouse/human) H chain. The insertion of the CD4 peptides was confirmed by nucleotide sequence analysis.

TABLE I

A		SFLT	KGPS	
B		GSFLT	KGPSK	LNDRA
C	GNQ	GSFLT	KGPS	
D	GNA	GSFLT	KGPS	
	↑ 38			↑ 55 of CD4

4. Examples

The engineering steps were as follows, with reference made to Figure 7. a) General structure of pNγ1CD4 expression vector modified to encode peptides 42-49$^{(A)}$ or 41-55$^{(B)}$ of human CD4. The CDR3 region of the host V_H (KAYSHG; residues 93-98) was mutagenized to introduce a single Kpnl/Asp718 site and yield the intermediate sequence KVPYSHG; residues 93-99. Here the amino acid 94A was deleted and substituted with the VP doublet which is encoded in the Asp718 cloning site. Subsequently, complementary oligonucleotides coding for peptides 42-49 or 41-55 of human CD4 sequence were introduced between 94V and 95P of the mutagenized V_H region. Only the coding oligonucleotide strands are shown here. The engineered V_HCD4^A or V_HCD4^B coded by the 2.3-kilobase (kb) EcoRI fragment was cloned upstream from a human γ1 constant (C) region gene contained in the 12.8 kb vector pNγ1. [Solazzo et al., Focus 10, 64 (1988). This is a PSV vector harboring a human γ_1 gene, encoded downstream from the EcoRI site. It also carries a neomycin resistance gene under the control of the SV40 promoter for the selection of stable transformant cells. See also Solazzo et al., Eur. J. Immunol. 19, 453 (1989).]

About 30 μg of the final DNA constructs pNγ1CD4^A or pNγ1CD4^B were electroporated in the murine J558L cell line (20 x 10^6 cells) using a field strength of 750 V/cm. Transfected cells were incubated without selection for 24

h and then selected in presence of 1.2 mg/ml G418 (GIBCO). G418-resistant clones secreting high level of the ^{Ag}Abs were identified by enzyme-linked immunosorbent assay (ELISA) using horseradish peroxidase (HRP)-conjugated goat antibody to human immunoglobulin (Ig) (SIGMA).

^{Ag}Abs were first concentrated by $(NH_4)_2SO_4$ precipitation and then purified by affinity chromatography on a Protein A (Pharmacia-LKB, Alameda, CA) column equilibrated with 3M NaCl/1M glycine, pH 8.9. Elution was performed using Glycine 0.1M- HCl/0.5M NaCl pH 2.8. The eluted fractions were neutralized using 1M Tris-HCl pH 8.0 and dialysed against phosphate-buffered saline (PBS). B, BamHI; RI, EcoRI; Neo, neomycin (G418) resistance; Amp, ampicillin resistance.

b) Schematic view of ^{Ag}Abs expressing CD4 peptides. The heavy chain is the fusion product of a human γ1C region with a murine V_H region engineered to express the CD4 sequences 42-49 and 41-55. The CDR3 region of the H chain was modified by inserting between 94V and 95P the residues SFLTKGPS(upper)$^{(A)}$ or GSFLTKGPSKLNDRA (lower)$^{(B)}$ sequences. The inserted sequences are flanked at each side by a VP doubet. The λ_1 light (L) chain is provided by the murine myeloma J558L used for transfection.

c) Recognition of the two ^{Ag}Abs by OKT4D a monoclonal antibody to CD4. ^{Ag}Abs were electrophored on a 10% Sodium dodecyl Sulfate (SDS)/Polyacrylamide Gel (PAGE) and 1.2 μg per lane were run under reducing conditions (5% β-mercaptoethanol). Protein were blotted onto 0.45-μm Polyvinylidene difluoride paper (PVDF) (Millipore). after blotting, the membrane was blocked by soaking in 5% dry milk in PBS and incubated for 1 hour at room temperature with OKT4D (2.5μg/ml), Pharmaceutical Research Institute-Raritan-NJ) a murine monoclonal antibody (IgG1,k) that binds residues 44-52 of human CD4. The bound antibody was revealed by ^{125}I-labeled goat antibody to mouse k. PVDF

paper was exposed to Kodak X-OMAT AR film for 3 days at -70 °C. A band in correspondence of the heavy chain is visible on the ^{Ag}Ab γ1CD4^{A} and ^{Ag}Ab γ1CD4^{B} but not in the γ1WT control.

The antigenized antibodies produced were expressed as transfectoma products obtained by introducing the appropriate plasmid vector (containing the engineered V domain) into J558L mouse myeloma cell line by electroporation. This cell line is a H chain-defective variant of myeloma J558, and carries the rearrangement for a λ1 light (L) chain. The overall structure of the antibodies that are obtained by this procedure is depicted in Figure 7. The supernatants of neomycin resistant colonies (stable transformants have been tested by enzyme-linked immunosorbent assay (ELISA) for immunoglobulin (Ig) production using goat antibodies to human Ig in a sandwich assay.

By this method, we selected cultures secreting ≥20 μg/ml. ^{Ag}Abs have been purified from culture supernatants by affinity chromatography on Protein A-Sepharose. The purified proteins have been concentrated and analyzed by SDS-PAGE for purity using Coomassie blue staining. Verification of the expression of the CD4 inserts has been done by solid phase radioimmunoassay (RIA) and Western blot using OKT4D, a monoclonal antibody to CD4 whose recognition site is around amino acid residue 47. Table II indicates that the four ^{Ag}Abs^{CD4} all bound in a dose dependent manner glycosylated, recombinant gp120 in a solid-phase assay, hence suggesting that the residues grafted into the host antibody molecule are sufficient to mediate binding. A reference HIV^{+} serum was used as a positive control.

TABLE II

Antibodies Antigenized with Peptide Structures of Human CD4 Bind Recombinant gp120 in ELISA

Antibody	CD4 Residues		ELISA O.D. (A_{492})		
			100 (μg/ml)	25 (μg/ml)	6.25 (μg/ml)
γ1CD4	A	42-49	1435	976	686
"	B	41-55	1124	722	541
"	C	38-49	1401	1110	817
"	D	$38\text{-}49^{Q10A}$	1137	1093	962
γ1WT			618	496	476
HIV^+ serum (1:1000)			1283		
HIV^- serum (1:1000)			525		

The first two $^{Ag}Abs^{CD4}$ were tested for their ability to induce site-specific and biologically-active antibodies to CD4. In particular, we intended to generate antibodies against the HIV interactive site of CD4 that could block syncytia formation. Precisely, we used a rapid method for the formation and analysis of syncytia *in vitro*. This test utilizes $CD4^-$ 8E5 T cells (transfected with a HIV defective for reverse transcriptase) and $CD4^+$ human T cells MOLT3. When these two cells are incubated for 3 hours at 37°C, syncytia form in large number and these can be quantified by visual inspection.

8E5 ($1.2\ 10^6$/ml) and MOLT3 (2×10^6/ml) cells were mixed in equal volumes in a final volume of 300 μl, plated in 96-well plate and incubated at 37°C for 30 minutes. The plate was then spun for 5 minutes at 1200 rpm and incubated at 37°C for an additional 3 hours. At the end of the incubation the cells were transferred to a flat-bottom 96-well plate and the formation of syncytia recorded.

Rabbits were immunized at regular intervals for a total of 5 injections. Sera were taken 10 days after the

immunizing injection. Only rabbits immunized with ^{Ag}AbCD4^{B} (residues 41-55) produced syncytia-inhibiting antibodies (Figure 7). By FACS, 2 out of 5 bound, to some extent, to CD4. CD4 spontaneously binds Ig of unrelated specificity, thereby making it difficult to discriminate between paratope-dependent and paratope-independent binding. See Table III.

TABLE III

NZW Rabbits
100 μg/injection
x5 subcutaneously

Rabbit #	Immunogen	FACS (CEM cells)	Syncytia Inhibition (%) **
1	γ1CD4$^{42-49}$	-	5
2	"	-	0
3	"	-	0
4	"	-	0
5	"	-	8
6	γ1CD4$^{41-55}$	+	45
7	"	+	28
8	"	-	30
9	"	-	35
10	"	-	0

* sera were used at the dilution of 1:500
** Inhibition was evaluated as follows:
% of inhibition postimmune serum - % of inhibition preimmune serum

Similarly, mice were immunized using only ^{Ag}AbCD4^{B} and obtained syncytia inhibiting antibodies in every instance. From a mouse with the highest titer (1:500) several hybridomas were generated and screened using the microsyncytia assay. Out of many positive clones, we retained four (Figure 8). All were of the IgG1, k isotype and inhibited when diluted 1:2 with values ranging from 45 to 82 %. The 4 clones were further subcloned, purified and characterized. As an example, we have displayed the results obtained with one of them (mAbPL1) (Figure 9).

The upper panel shows a dose-dependent effect of this antibody on syncytia formation. In the lower panel, a FACS profile on CEM cells is shown using mAbPL1 and by comparison Leu3a. Antibody PL1 bound with a unimodal peak, but with intensity lower than Leu3a. All antibodies also bound with CD4 in Western blot and the immunizing ^{Ag}Ab (Figure 9).

Taken together, these results indicate that ^{Ag}Ab$^{CD4\text{-}B}$ efficiently elicited antibodies against the site of CD4 engineered into the Ig V_H domain, further indicating that the conformation retained by the CD4 14^{mer} integrated within the Ig loop is adequate for immunogenicity. Thus, this method appears to provide an ideal solution to the limitation of immunogenicity of synthetic peptides.

5. References

The following references are grouped by number referring to parenthetical numbering in the preceding text. Each of these documents is hereby expressly incorporated by reference herein:

1. J. Arthos, *et al.*, Cell 57, 469-481 (1989).

2. L. K. Clayton, *et al.*, Nature 335, 363-366 (1988).

3. L. K. Clayton, M. Sieh, D. A. Pious, E. L. Reinherz, Nature 39, 548-551 (1989).

4. ID. Lamarre, *et al.*, Science 245,743-746 (1989).

5. T. Mizukami, T. R. Fuerst, E. A. Berger, B. Moss, Proc. Natl. Acad. Sci. USA 85, 9273-9277 (1988).

6. A. Peterson, B. Seed, Cell 54, 65-72 (1988).

7. G. S. Wood, N. L. Warner, R. A. Warnke, J. Immunol. 131, 12-216 (1983).

8. D. R. Lucey, D. I. Dorsky, A. Nicholson-Weller, P. F. Weller, J. Exp. Med. 169, 327-332 (1989).

9. S. J. Stewart, J. Fujimoto, R. Levy, J. Immunol. 136, 3773-3778 (1986).

10. W. E. Biddison, P. E. Rao, M. A. Tale, G. Goldstein, S. Shaw, J. Exp. Med. 156, 1065-1076 (1982).

11. D. Gay, *et al.*, Nature 328, 626-629 (1987).

12. S. C. Meuer, S. F. Schlossman, E. L. Reinherz, Proc. Natl. Acad. Sci. USA 79, 4395-4399 (1982).

13. S. L. Swan, D. P. Dialynas, F. F. W., M. English, J. Immunol. 132, 1118-1123 (1984).

14. F. Emmrich, Immunol. Today 9, 296-300 (1988).

15. A. Veillette, M. A. Bookman, E. M. Horak, L. E. Samelson, J. B. Bolen, Nature 338, 257-259 (1989).

16. I. Bank, L. Chess, Exp. Med. 162, 1294-1303 (1985).

17. M. L. Blue, *et al.*, J. Immunol. 140, 376-383 (1988).

18. E. G. Engleman, C. J. Benike, C. Metzler, P. A. Gatenby, R. Evans, J. Immunol. 130, 2623-2628 (1983).

19. B. Sleckhann, *et al.*, Nature 328, 351-353 (1987).

20. C. E. Rudd, J. M. Trevillyan, J. D. Dasgupta, L. L. Wong, S. F. Schlossman, Proc. Natl. Acad. Sci. USA 85, 5190-5194 (1988).

21. A. Pelchen-Matthews, J. E. Armes, G. Griffiths, M. Marsh, J. Exp. Med. 173, 575-587 (1991).

22. P. Maddon, M. Littman, D. Maddon, L. Ches, R. Axe, Cell 42, 93-104 (1985).

23. Q. J. Sattentau, *et al.*, J. Exp. Med. 170, 1319-1334 (1989).

24. A. Ashkenazi, *et al.*, Proc. Natl. Acad. Sci. USA 87, 7150-7154 (1990).

25. S. E. Ryu, *et al.*, Nature 348, 419-426 (1990).

26. J. Wang, *et al.*, Nature 348, 411-418 (1990).

27. G. A. Schockmel, C. Somoza, S. J. Davis, A. F. Williams, D. Healey, J. Exp. Med. 175, 301-304 (1992).

28. M. H. Brodsky, M. Warton, R. M. Myers, D. R. Littman, J. Immunol. 144, 3078-3086 (1990).

29. V. S. Kalyanaraman, *et al.*, J. Immunol. 145, 4072-4078 (1990).

30. J. D. Lifson, *et al.*, Science 241, 712-716 (1988).

31. D. Healey, *et al.*, J. Exp. Med. 172, 1233-1242 (1990).

32. A. Truneh, *et al.*, J. Biol. Chem. 266, 5942-5948 (1991).

33. M. Ivey-Hoyle, *et al.*, Proc. Natl. Acad. Sci. USA 88, 512-516 (1991).

34. J. McKeating, P. Balfe, P. Clapham, R. A. Weiss, J. Virol. 65 4777-4785 (1991).

35. E. S. Daar, X. L. Li, T. Moudgil, D. D. Ho, Proc. Natl. Acad. Sci. USA 87, 6574-6578 (1990).

36. S. Turner, *et al.*, Proc. Natl. Acad. Sci. USA 89, 1335-1339 (1992.

37. J. P. Moore, J. A. McKeating, R. A. Weiss, Q. J. Sattentau, Science 250, 1139-1142 (1990).

38. E. A. Berger, J. D. Lifson, L. E. Eiden, Proc. Natl. Acad. Sci. USA 88, 8082-8086 (1991).

39. T. K. Hart, *et al.*, Proc. Natl. Acad. Sci. USA 88, 2189-2193 (1991).

40. J. S. Allan, Science 252, 1322-1323 (1991).

41. J. S. Allan, J. Strauss, D. W. Buck, Science 247, 1084-1088 (1990).

42. P. Lenert, D. Kroon, H. Spiegelberg, E. S. Golub, M. Zanetti, Science 248, 1639-1643 (1990).

43. S. Lederman, M. J. Yellin, A. M. Cleary, R. Gulick, L. Chess, J. Immunol. 144, 214-220 (1990).

44. R. T. Schooley, *et al.*, Ann. Intern. Med. 112, 247-253 (1990).

45. Lancet 335, 1128-1130 (1990).

46. A. G. Dalgleish, B. J. Thomson, T. C. Chanh, M. Malkovsky, R. C. Kennedy, Lancet ii, 1047-1050 (1987).

47. R. Attanasio, R. Kennedy, Intern. Rev. Immunol. 7, 109-119 (1990).

48. R. Attanasio, J. Allan, S. Anderson, T. Chanh, R. Kennedy, J. Immunol. 146, 507-514 (1991).

49. H. Zaghouani, *et al.* Proc. Natl. Acad. Sci. USA 88, 5645-5649 (1991).

50. D. Healey, L. Dianda, P. Beverley, J. Immunol. 148, 821-826 (1992).

51. Capon, D, *et al*. Nature 337, 525 (1989).

52. Clapham, P., *et al*. Nature 337, 368 (1989)

53. Deen, K. *et al*. Nature 331, 82 (1988)

54. Fisher, R. *et al*. Nature 331, 76 (1988)

55. Hussey, R. *et al*. Nature 331, 78 (1988)

56. Smith, D. *et al*. Science 238, 1704 (1987)

57. Traunecker, A. *et al*. Nature 331, 84 (1988)

The foregoing description details specific methods that can be employed to practice the present invention. Having detailed specific methods initially used to identify, isolate, characterize, prepare and use the immunoglobulins hereof, and a further disclosure as to specific model entities, the art skilled will well enough know how to devise alternative reliable methods for arriving at the same information and for extending this information to other intraspecies and interspecies related immunoglobulins. Thus, however detailed the foregoing may appear in text, it should not be construed as limiting the overall scope hereof; rather, the ambit of the present invention is to be governed only by the lawful construction of the appended claims.

Claims:

1. An immunoglobulin molecule containing at least one CD4 HIV binding domain within the third complementarity-determining region (CDR) in the N-terminus variable domain thereof, said immunoglobulin molecule having the effector function conferred by the constant region of the immunoglobulin, and having specific CD4 epitope reactivity.

2. An immunoglobulin according to Claim 1 wherein said binding domain is the region of amino acid residues selected from the group consisting of 42 to 49, 41 to 55, and 38 to 49.

3. As a product of recombinant DNA technology, an immunoglobulin according to Claim 1.

4. A heavy chain of an immunoglobulin containing within the third complementarity-determining region (CDR) in the N-terminus variable domain thereof at least one CD4 HIV binding domain.

5. As a product of recombinant DNA technology, the heavy chain according to Claim 3.

6. The heavy chain according to Claim 4 in a form unassembled with its counterpart heavy chain.

7. The heavy chain according to Claim 6 in a form unassembled with its associated light chain.

8. A chimeric immunoglobulin molecule according to Claim 1.

9. The chimeric immunoglobulin molecule according to Claim 7 comprising hybrid heavy chain comprising both human and non-human sequences.

10. A pharmaceutical composition containing as an essential principle an immunoglobulin molecule according to Claim 1.

11. The composition according to Claim 10 suitable for administration to a human subject.

12. The composition according to Claim 10 in the form of an administrable vaccine.

13. A DNA molecule that is a recombinant DNA molecule or a cDNA molecule encoding an immunoglobulin molecule according to Claim 1.

14. An expression vector operatively harboring DNA encoding an immunoglobulin, defined according to Claim 13.

15. A recombinant host cell transfected with an expression vector according to Claim 14.

16. A process of preparing an immunoglobulin molecule according to Claim 1 which comprises expressing in a recombinant host cell transfecting DNA encoding said immunoglobulin molecule.

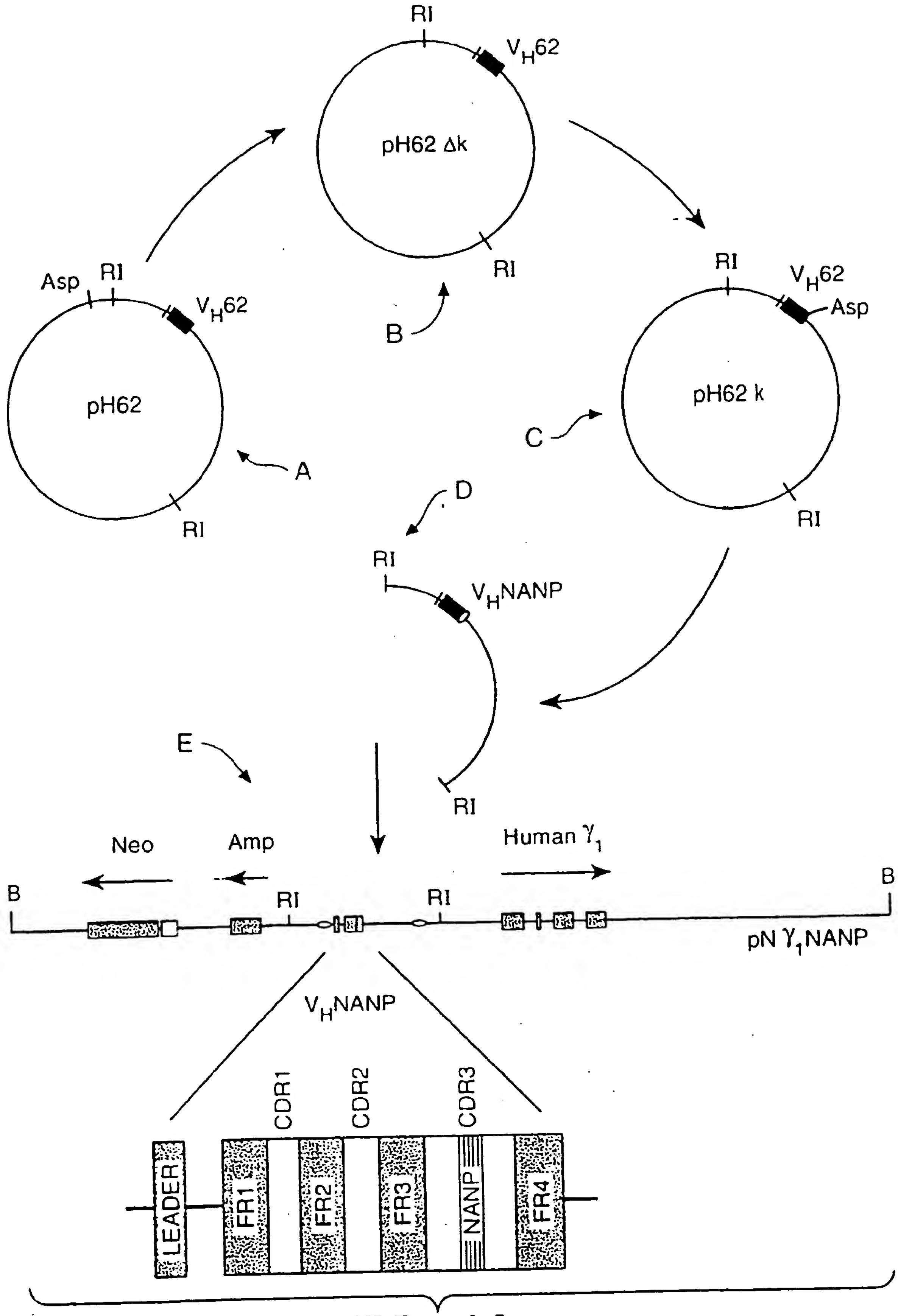

RI
V_H62
pH62 Δk
RI
Asp
RI
V_H62
pH62
RI
A
B
RI
V_H62
Asp
pH62 k
RI
C
D
RI
V_HNANP
RI
E
Neo
Amp
Human γ_1
B
RI
RI
B
pN γ_1NANP
V_HNANP
CDR1
CDR2
CDR3
LEADER
FR1
FR2
FR3
NANP
FR4

FIG._1A

pH62Ak

90

```
          |<--------- CDR3 ----------------->|
C   A   R   K   A   Y   S   H   G   M   D   Y   W
TGT GCA AGA AAG GCC TAC TCT CAT GGT ATG GAC TAC TGG
```

pH62k

90

```
          |<--------- CDR3 --------------------->|
C   A   R   K   V   P   Y   S   H   G   M   D   Y   W
TGT GCA AGA AAG GTA CCC TAC TCT CAT GGT ATG GAC TAC TGG
                Asp718
```

pH *NANP*

90

```
          |<--------- CDR3 --------------------------------------------------->|
C   A   R   K   V   P    N   A   N   P     V   P   Y   S   H   G   M   D   Y   W
TGT GCA AGA AAG GTA CCC [AAT GCA AAC CCA]3 GTA CCC TAC TCT CAT GGT ATG GAC TAC TGG
```

FIG._1B

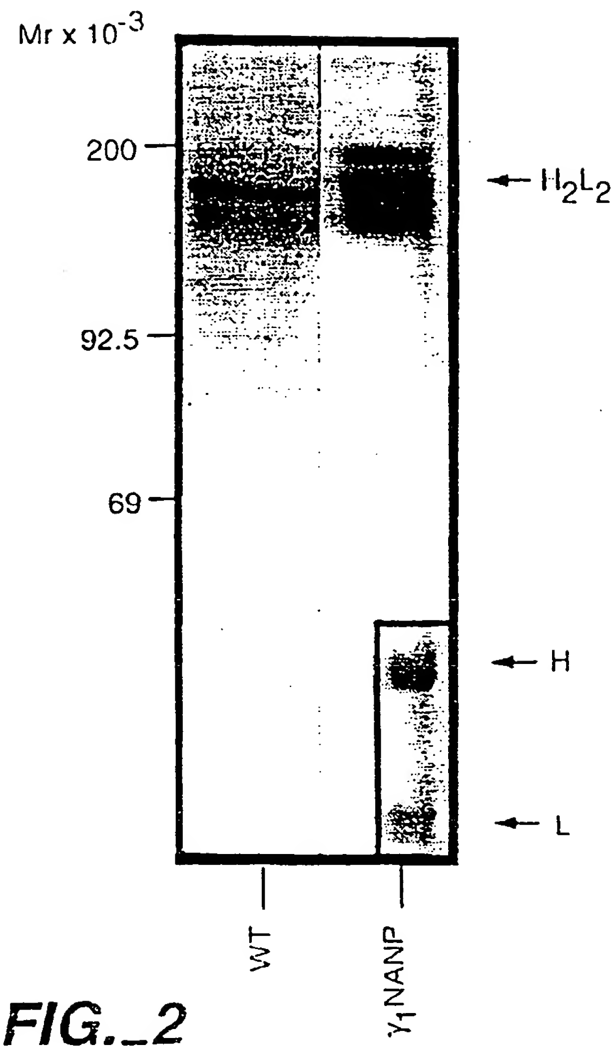


FIG._2

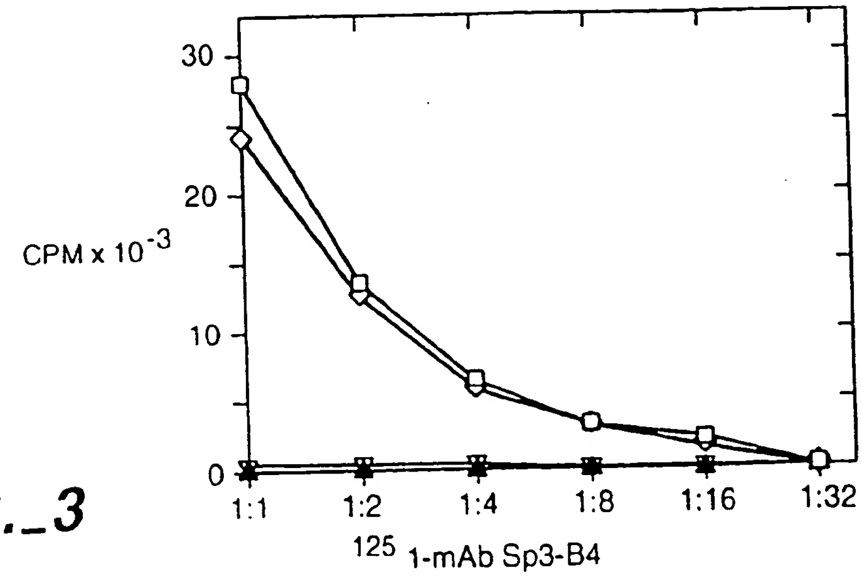


FIG._3

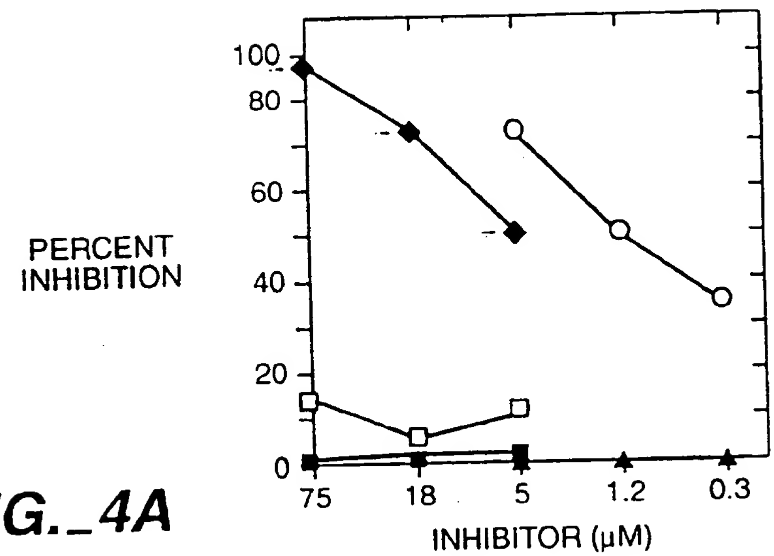


FIG._4A

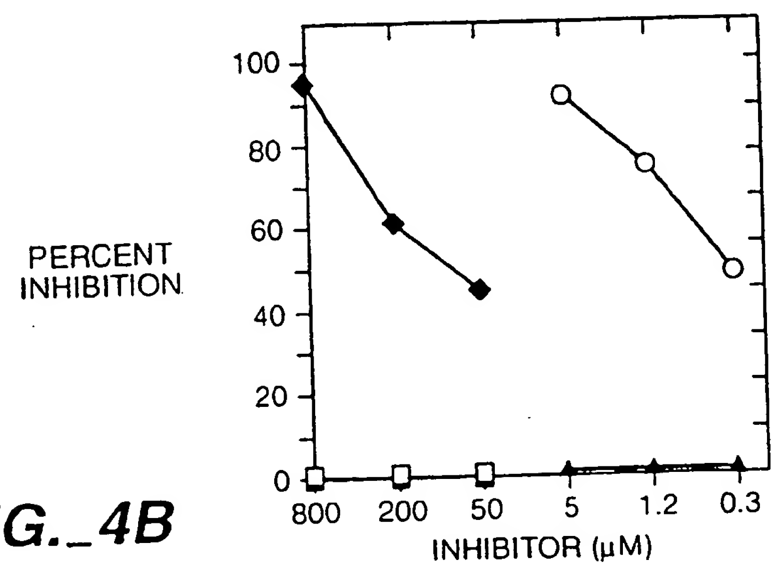
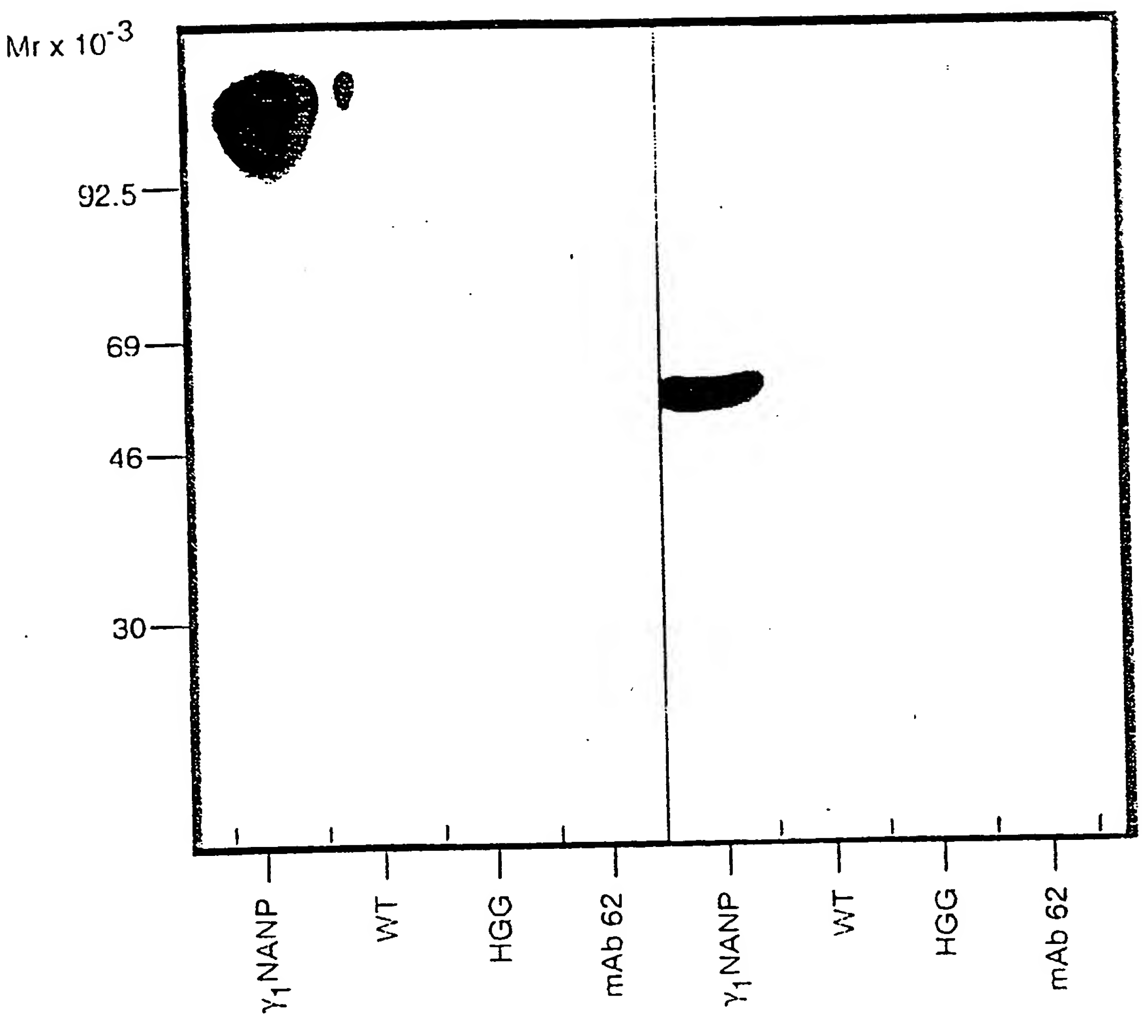


FIG._4B



Mr x 10^{-3}
92.5
69
46
30
γ_1NANP
WT
HGG
mAb 62
γ_1NANP
WT
HGG
mAb 62

FIG._5

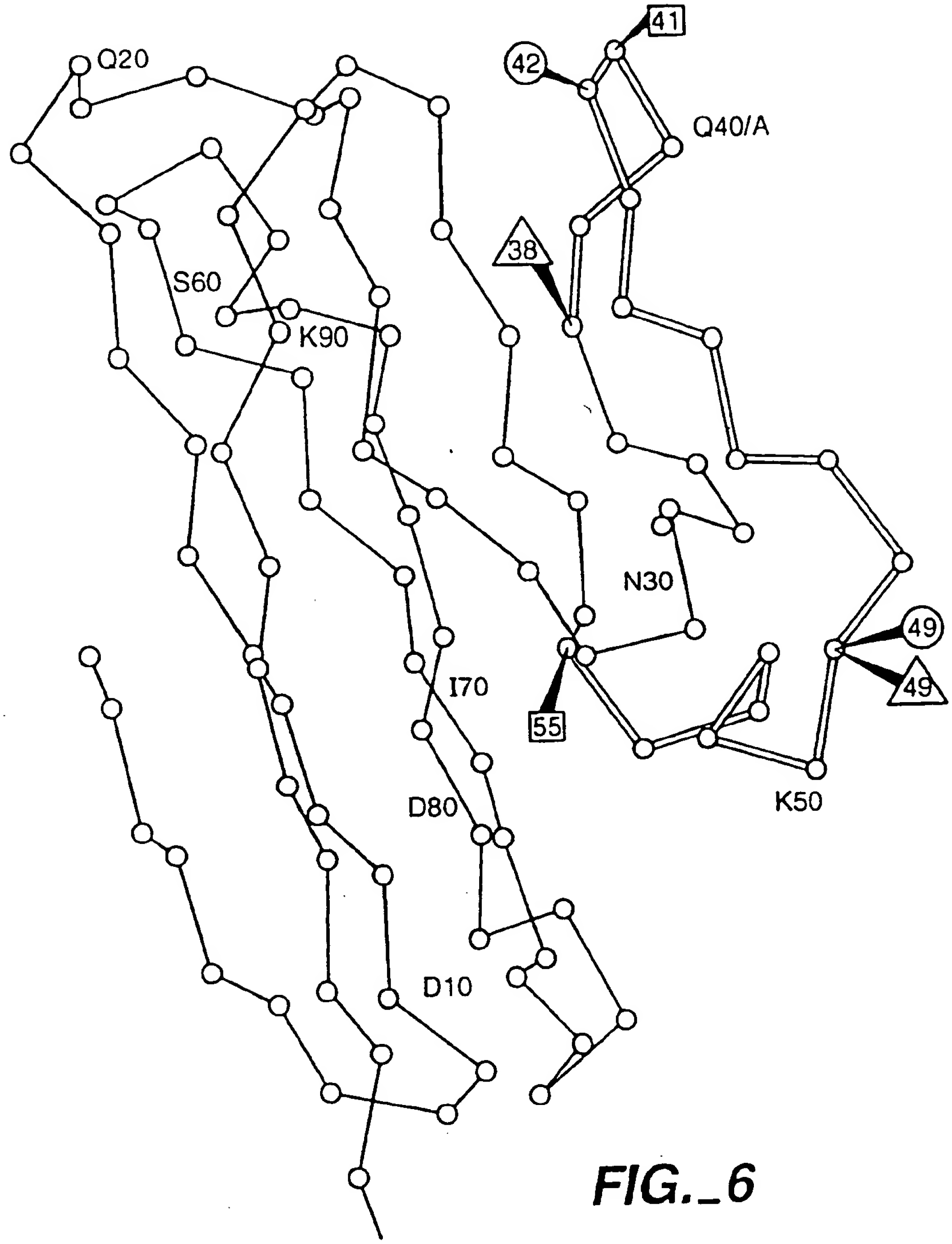


FIG._6

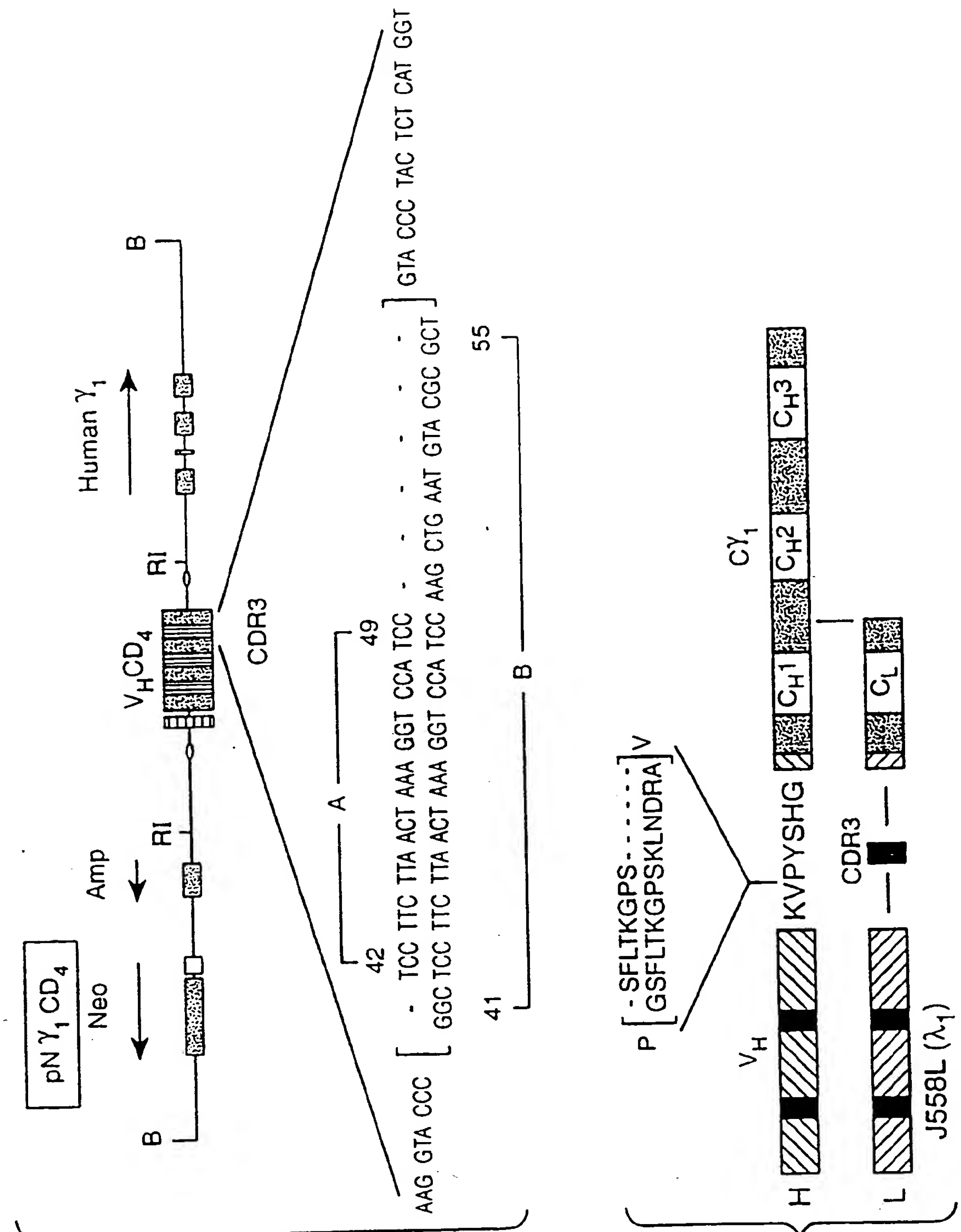

pN γ_1 CD$_4$
Neo
Amp
RI
V_HCD$_4$
RI
Human γ_1
B
B
CDR3
AAG GTA CCC
- TCC TTC TTA ACT AAA GGT CCA TCC - - - - - - - GTA CCC TAC TCT CAT GGT
GGC TCC TTC TTA ACT AAA GGT CCA TCC AAG CTG AAT GTA CGC GCT
42
A
49
41
B
55
P
-SFLTKGPS- - - - - -
GSFLTKGPSKLNDRA
V
V_H
KVPYSHG
$C\gamma_1$
C_H1
C_H2
C_H3
H
L
CDR3
C_L
J558L (λ_1)

FIG._7A

FIG._7B

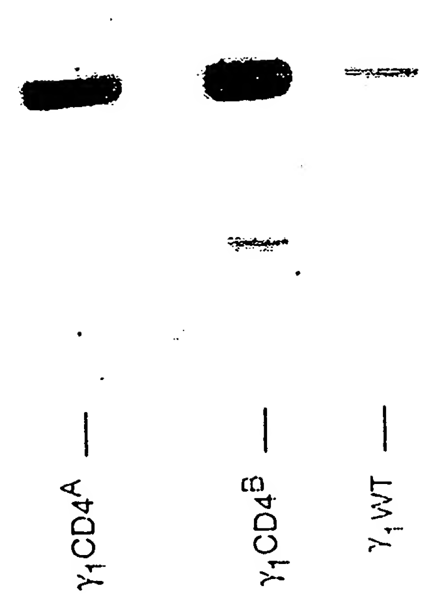

γ_1CD4^A
γ_1CD4^B
γ_1 WT

FIG._7C


mAb PL1
mAb PL2
mAb PL4
mAb PL5
OKT4D
CONTROL

FIG._10

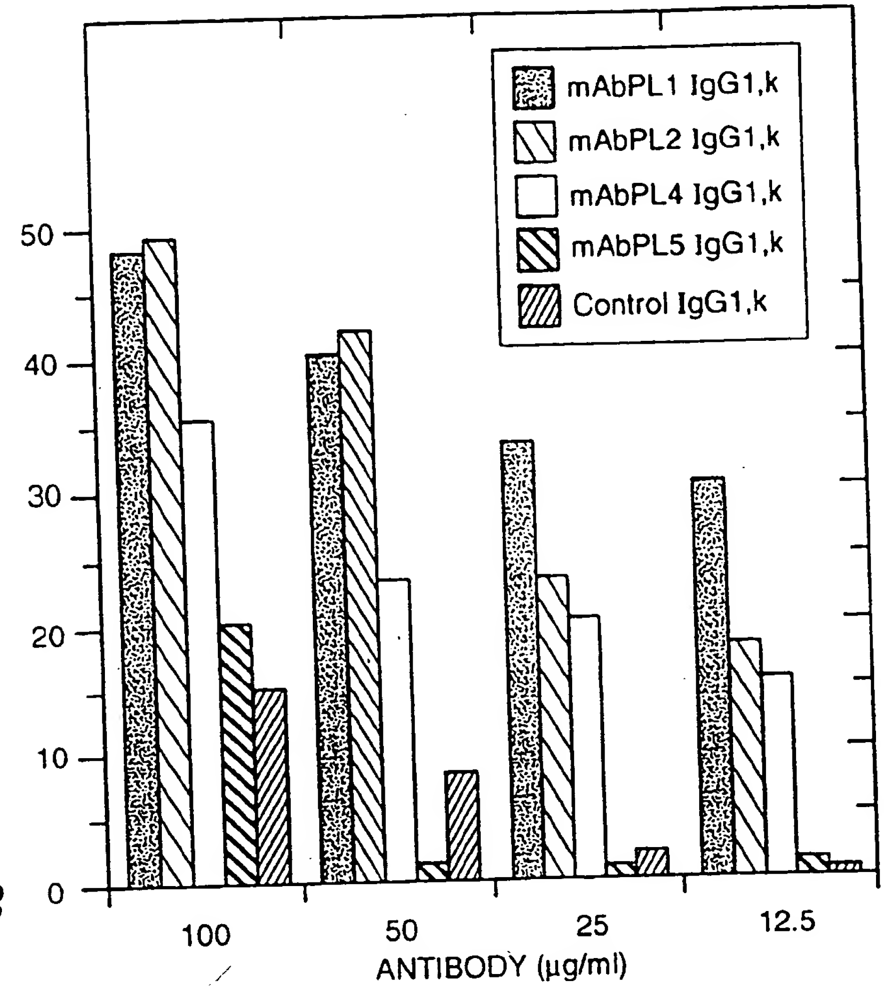

mAbPL1 IgG1,k
mAbPL2 IgG1,k
mAbPL4 IgG1,k
mAbPL5 IgG1,k
Control IgG1,k
PERCENT OF INHIBITION
0
10
20
30
40
50
100
50
25
12.5
ANTIBODY (μg/ml)

FIG._8

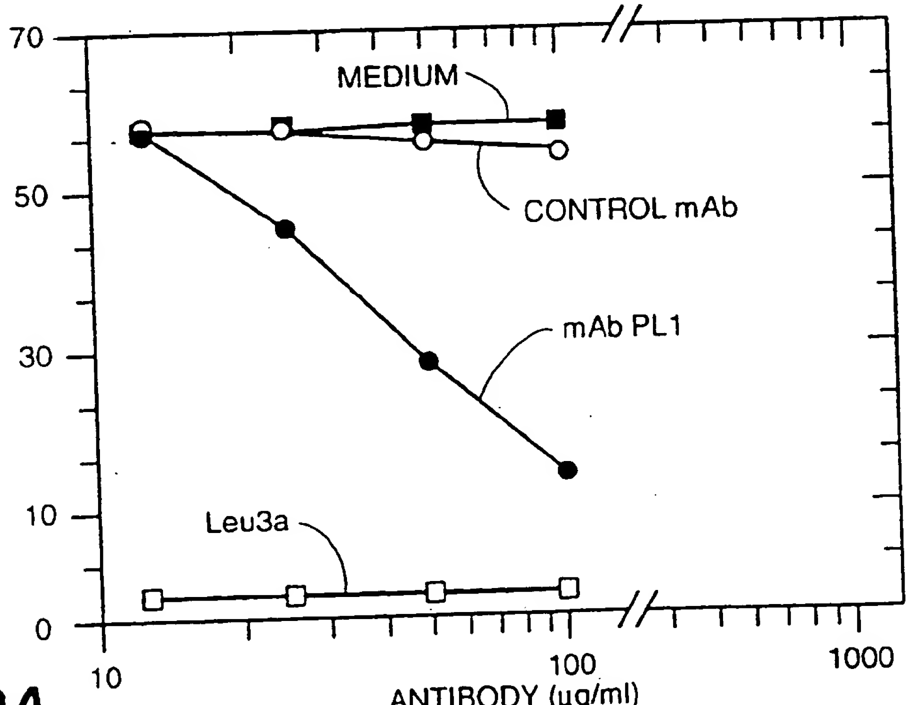

MEDIUM
CONTROL mAb
mAb PL1
Leu3a
NUMBER OF SYNCYTIA
0
10
30
50
70
10
100
1000
ANTIBODY (μg/ml)

FIG._9A

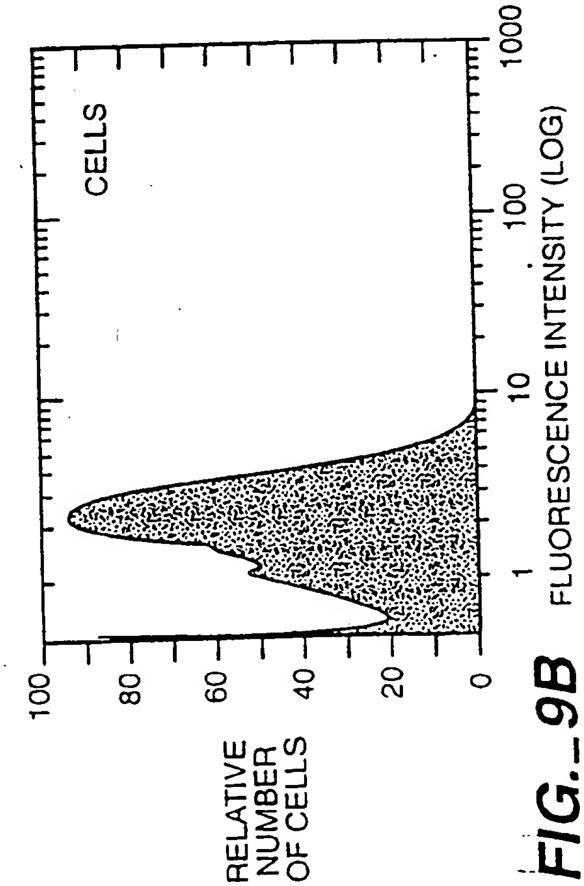


FIG._9B

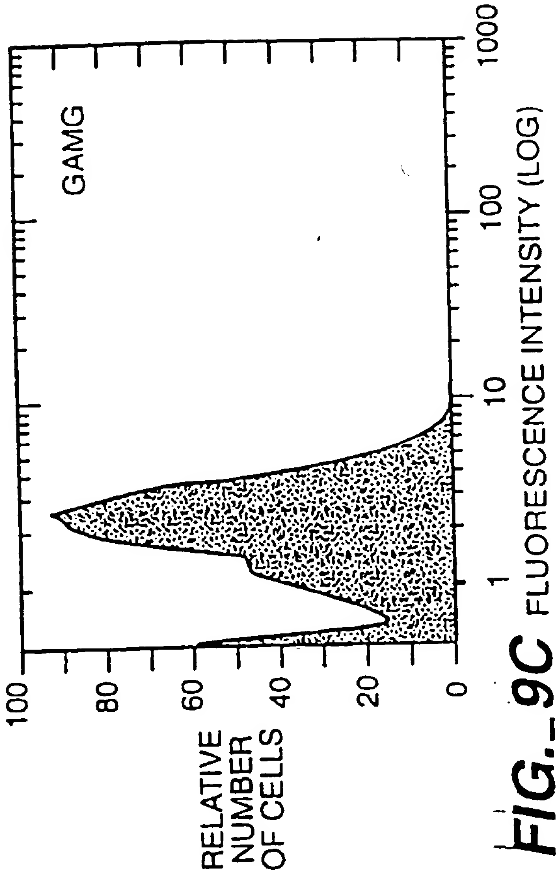


FIG._9C

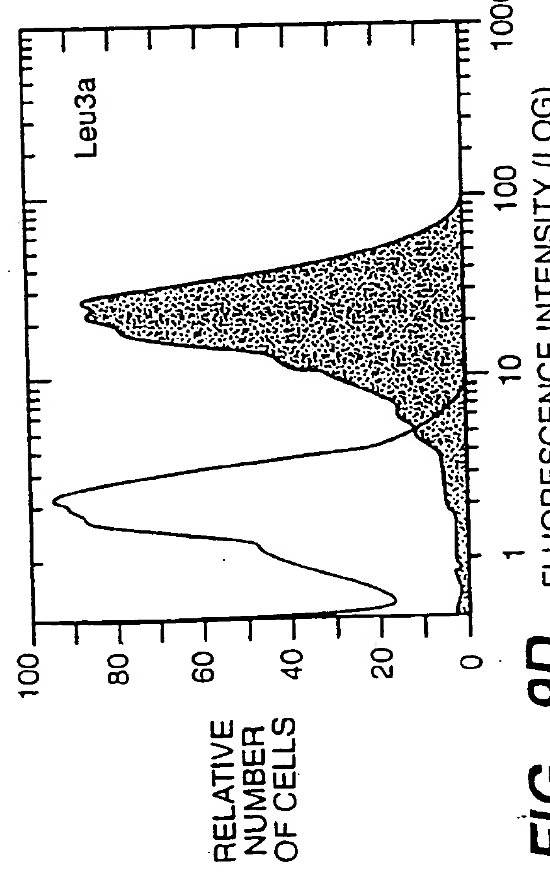


FIG._9D

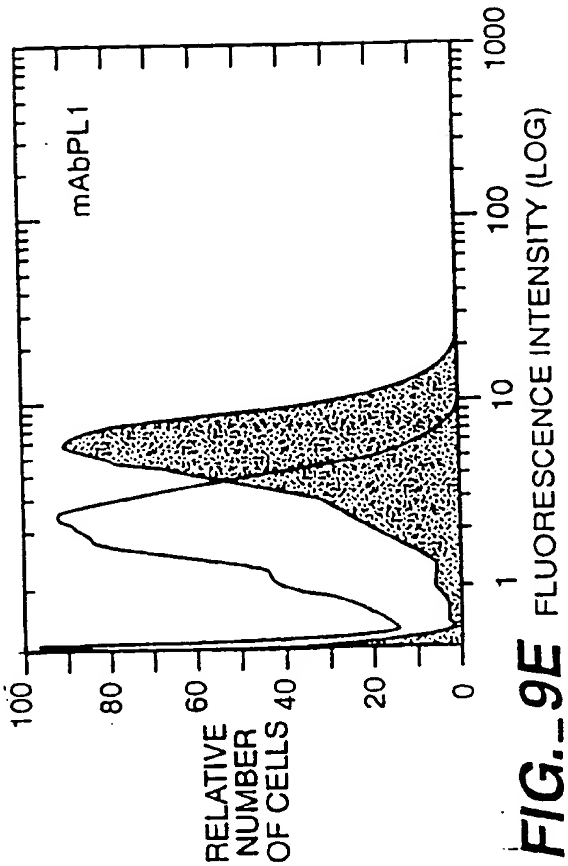


FIG._9E

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/12339

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :530/387.3; 536/25.23; 435/69.6, 320.1; 424/85.8.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/387.3; 536/25.23; 435/69.6, 320.1; 424/85.8.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings of the National Academy of Sciences, Volume 88, issued June 1991, Billetta et. al. "Immunogenicity of an Engineered Internal Image Antibody", pages 4713-4717, see especially pages 4713-4715.	1-16
Y	The Journal of Biological Chemistry, Volume 266, No. 9, issued 25 March 1991, Truneh et. al., "A Region in Domain 1 of CD4 Distinct From the Primary gp1220 Binding Site Is Involved In HIV Infection and Virus-mediated Fusion*", pages 5942-5948, see entire article.	1-16

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search 31 MARCH 1994	Date of mailing of the international search report 14 APR 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. Not Applicable	Authorized officer Lila Feisee Jill Warden for Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	The FASEB Journal, Volume 6, No.5, issued 28 February 1992, Billetta et. al, "Immunogenicity of Hydrophylic Sequences In Antegenized Antibodies", No. 6226, see entire abstract.	1-16
Y	Immunomethods, Volume 1, issued 1992, Billetta et. al., "Ligand Expression Using Antigenization of Antibody: Principle and Methods", pages 41-51, see especially pages 44-48.	1-16

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

C07K 15/28; C12P 21/06; A61K 39/395; C12N 15/00, 1/00, 5/00; C07H 15/00.